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The Inhibitory Effect of a Neuropeptide, *Manduca*FLRFamide, on the Midgut Activity of the Sphingid Moth, *Agrius convolvuli*

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ABSTRACT—The effect of *Manduca*FLRFamide, a neuropeptide originally isolated from the central nervous system of a sphingid moth, *Manduca sexta,* was examined on the midgut motility of another sphingid moth, *Agrius convolvuli.* The peptide was shown to suppress the spontaneous contractions of the isolated gut in adult *Agrius* at low concentrations ($\geq 10^{-10}$ M). Immunoreactivity against anti-FMRFamide antiserum was also detected in the brain-suboesophageal ganglion and the retrocerebral neurohaemal organs of the moth. These observations suggest that *Manduca*FLRFamide, or its closely-related analog, is a neuropeptide which regulates the activity of the digestive tract in *Agrius.*

INTRODUCTION

*Manduca*FLRFamide (pQDVVHSFLRF-NH_2 is a neuropeptide originally isolated from the brain-suboesophageal ganglion (SOG) of the tobacco hawkmoth, *Manduca sexta* [6]. This peptide was also found in the brain-attached neurohaemal organs, corpora cadiaca and corpora allata (CC-CA), and has therefore been supposed to act as a circulating hormone. However, little is known about its actual target organs; the only known bioactivity for *Manduca*FLRFamide is the potentiation of the neurally-evoked contraction of the flight muscle of the moth [6]. In contrast, physiological functions of other FMRFamide-family peptides in insects, such as SchistoFLRFamide [8] and leucomyosuppressin [1], have been extensively investigated. Their possible targets are not only skeletal muscles [8], but also various visceral organs, such as the heart [8], oviduct [7] and fore- and hindguts[1]. These findings encouraged us to postulate that *Manduca*FLRFamide is involved in the regulation of visceral organs in the moth.

Here we show that *Manduca*FLRFamide has a potent inhibitory effect on the midgut spontaneous contractions of the moth, *Agrius convolvuli. Agrius* is a sphingid moth closely related to *Manduca* which has recently been established as an experimental insect substituting for *Manduca* [5]. We also demonstrate the FMRFamide-like immunoreactivity in the brain-SOG and the CC-CA of *Agrius,* suggesting that *Manduca*FLRFamide, or a very similar peptide, may act as a neurohormone in the moth.

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MATERIALS AND METHODS

Animals

Larvae of *Agrius convolvuli* were reared on

artificial diet [6] in a constant environment (27°C, ≈60% relative humidity, 16 hr light: 8 hr dark photoperiod). Adult animals 1–3 days after eclosion were used in experiments. They were fed on 25% sugar water, and kept at 25°C and 75–80% relative humidity with a 16 hr light: 8 hr dark cycle.

Measurement of midgut activity

Animals were anesthetized with CO_2 gas and the abdomen was removed. An incision was made along the dorsal midline and the midgut was carefully isolated. After removing the Malpighian tubules and trachea, both ends of the midgut were tied with thread. The gut preparation was suspended in an experimental chamber (1.2 ml) and continuously perfused with physiological saline at 0.7 ml/min. The composition of the saline was essentially the same as Truman's [9]; 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2$, 3 mM $CaCl_2$, 2.5 mM Na-phosphate buffer, 150 mM glucose, pH 6.5. Spontaneous contractions were monitored through a tension transducer. Midgut activity was calculated as the sum of the peaks in tension of contractions recorded over a 5 min period. Peptide samples were dissolved in the saline and perfused for 10 min. Bioactivity of the peptide was expressed as a percentage of control midgut activity.

Immunohistochemistry

The brain-SOG and the CC-CA complex were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.15 M NaCl/0.1 M phosphate buffer, pH 7.2) for 4–5 hr at 4°C. After washing with PBS several times, the tissues were incubated in rabbit anti-FMRFamide antiserum (1:200) diluted with PBS containing 2% Triton X-100 and 1% bovine serum albumin for 48 hr at 10°C. The tissues were then placed in 5% normal goat serum/PBS for 2 hr. The incubation with the rhodamine-conjugated secondary antibody (1:100) was for 3–4 hr at 10°C and was followed by several PBS washes. The tissues were then dehydrated, cleared in methyl salicylate and examined on the light microscope.

The specificity of the immunostaining was confirmed by preabsorption experiments using 1 mM synthetic *Manduca*FLRFamide.

Chemicals

Anti-FMRFamide antiserum was purchased from Peninsula Laboratories Inc. and the rhodamine-conjugated anti-rabbit IgG antibody, from Kirkegaard & Perry Laboratories Inc. *Manduca*FLRFamide and FLRFamide were synthesized using a peptide synthesizer (Applied Biosystems Inc., 431A). FMRFamide, proctolin and leucopyrokinin were purchased from Sigma, and YGGFMRFamide from Peninsula.

RESULTS

In the continuous-perfusion system, the isolated midgut preparation showed spontaneous rhythmic contractions for more than 12 hr, which made it possible to examine the bioactivity of multiple samples quantitatively. Using this system, it was shown that *Manduca*FLRFamide potently suppressed the midgut activity in a dose-dependent manner (Fig. 1). The threshold concentration was around 10^{-10} M. The complete arrest of spontaneous activity was observed at 3×10^{-10} M or higher, and its duration depended on doses; for example, 8 min and 22 min at 3×10^{-10} M and 10^{-9} M, respectively, in the preparation shown in Figure 1. The inhibitory effect was reversible, although recovery was slow. In addition to *Manduca*FLRFamide, three other FMRFamide-related peptides (FaRPs), FMRFamide, FLRFamide and YGGFMRFamide, were also tested for their effects on the midgut. All the three peptides showed similar inhibition but were 10^2–10^4-fold less potent than *Manduca*FLRFamide (Fig. 2). Proctolin and leucopyrokinin, hindgut stimulators in the cockroach, were also tested and found to be inactive even at 10^{-5} M (data not shown).

In order to confirm that *Manduca*FLRFamide or its counterpart is found in the central nervous system of *Agrius*, in addition to that of *Manduca*, we localized the peptide using immunohistochemical techniques with anti-FMRFamide antiserum. In the whole-mount preparation of the brain-SOG, a number of immunoreactive neuronal cells were located mainly at the dorsal surface of the brain, i.e. the pars intercerebralis and pars lateralis (Fig. 3A), and at the anterior surface of

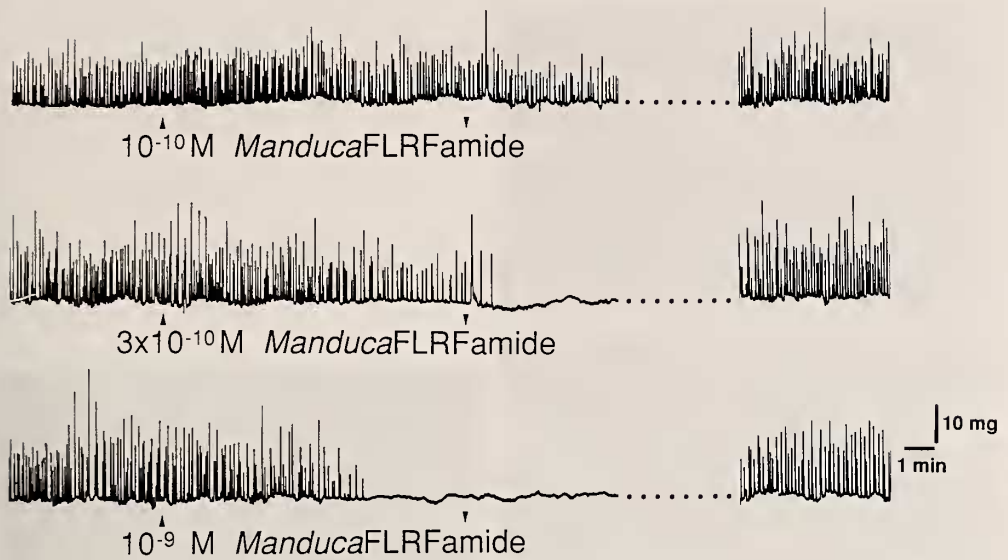

FIG. 1. Inhibitory effect of *Manduca*FLRFamide on the spontaneous contractions of *Agrius* midgut. Upward arrowheads indicate the time when the peptide solution reached the experimental chamber. Downward arrowheads indicate the time when the normal saline reached the chamber. Recovery after washout is shown to the right; each trace was recorded, respectively, 17 min, 25 min and 52 min after the washout had started. All the traces were recorded from the same preparation.

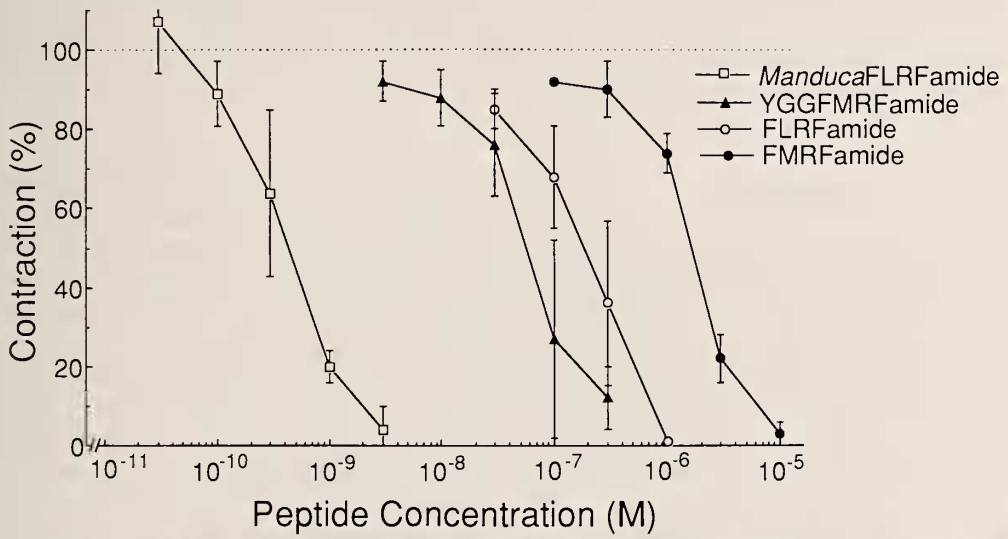

FIG. 2. Dose-response relationship for *Manduca*FLRFamide and other FaRPs. Vertical axis, "Contraction (%)", was calculated by [sum of peak tension during 5 min in the presence of peptide (5 min–10 min after the peptide reached the chamber)]/[sum of peak tension during 5 min just prior to the addition of peptide]×100. The data are mean±SE of 3–4 preparations.

the SOG (Fig. 3B). On the other hand, in the CC-CA, no cell bodies were stained but immunoreactive fibers with many varicosities were observed throughout the CC-CA (Fig. 3C). All the immunoreactivity detected was completely eliminated when the antiserum had been preabsorbed with 1 mM *Manduca*FLRFamide (data not shown).

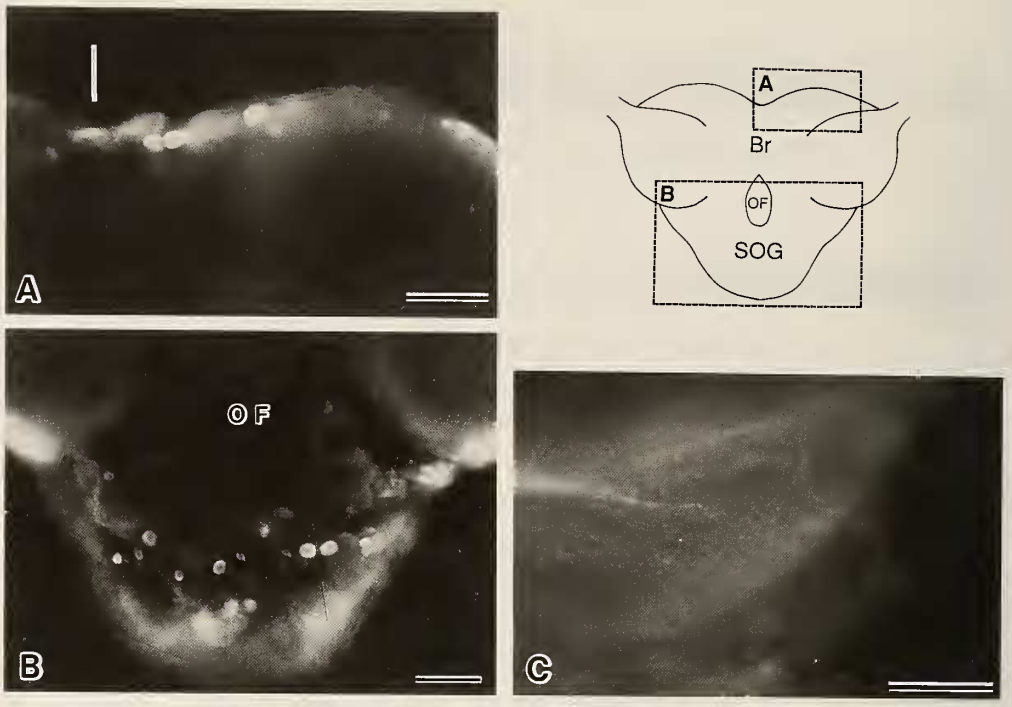

Fig. 3. FMRFamide-like immunoreactivity in the brain, SOG and CC-CA of an adult *Agrius*. A. Frontal view of the dorsal part of the brain. Vertical line indicates the midline of the brain. Scale, 100 μm. B. Anterior surface of the SOG. Scale, 100 μm. C. Immunoreactive fibers with many varicosities in the CA. Scale, 50 μm. Inset. Frontal view of the brain and SOG. Dotted rectangles marked A and B correspond to the photographs A and B. Br, brain; SOG, suboesophageal ganglion; OF, oesophageal foramen.

DISCUSSION

We have shown that *Manduca*FLRFamide potently suppressed the spontaneous contractions of the midgut of *Agrius* with a low threshold concentration of 10^{-10} M. This is the first demonstration that *Manduca*FLRFamide shows an effect not only on a skeletal muscle [6] but also on a visceral organ in the moth at very low concentrations. The effect was dose dependent, reversible and extremely potent compared to other FaRPs such as FMRFamide, FLRFamide and YGGFMRFamide, implying a physiological role for the peptide in *Agrius*. We also demonstrated that the FMRFamide-like immunoreactivity, which was eliminated by preabsorption with *Manduca*FLRFamide, was localized in the brain-SOG and the CC-CA of *Agrius*. The distribution pattern of the immunoreactive cells in the brain-SOG is similar to that in *Manduca,* where a number of FMRFamide-like immunoreactive neuronal cells are known to send their axons to the CC-CA [2, 3]. These observations, together with the fact that *Manduca*FLRFamide was originally isolated from the brain-SOG and the CC-CA of *Manduca,* suggest that *Agrius* also possesses *Manduca*FLRFamide or at least its closely related peptide in the brain, SOG, and CC-CA. In addition, the observation of many varicosities in the CC-CA of *Agrius* suggest that the immunoreactive material might be released from these neurohaemal organs into the systemic circulation. In larval lepidoptera, it has been reported that the haemolymph level of FMRFamide-like immunoreactivity is increased in the starved animal [4]. Although the nature of this immunoreactivity has not been identified, it is possible that *Manduca*FLRFamide, or its counterpart, acts as a circulating hormone to regulate the activity of the digestive tract in the adult moth. The physiological mechanisms by which *Man-*

*duca*FLRFamide and its counterparts regulate the digestive system throughout the life cycle of lepidopteran insects are to be clarified in further investigation.

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